SEPARATION OF MYCOBACTERIAL ANTIGENS BY ISOELECTRIC FOCUSING AND EVALUATION OF THEIR IMMUNOGENIC PROPERTIES

A THESIS

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LIST OF ABBREVIATIONS

ABTS	2.2'- Azino-di-3-ethylbenzthiazoline Sulfonate (Substrate Powder)
BSA	Bovine Serum Albumin
CE	Cell Extract
DEAE	Diethylaminoethyl Sephadex
ELISA	The Enzyme-Linked Immunosorbent Assay
IEF	Isoelectric focusing
IgG	Immunoglobulin G
IWGMT	International Working Group of Mycobacterial Taxonomy
MAI	*Mycobacterium avium - intracellulare* complex
PAG	Polyacrylamide gel
PBE 94	Polybuffer exchanger
PBS	Phosphate Buffer Saline
pI	Isoelectric Point
SDS	Sodium Dodecyl Sulfate
TCA	Trichloroacetic Acid

CHAPTER I

INTRODUCTION

In these studies effort was made to detect possible specific antigens of different mycobacterial strains which could be useful as skin test antigens and permit early detection of a mycobacterial infection. These studies could also offer information on the various antigens, both shared and unshared, that may confer immunity against either a homologous or a heterologous infection. Both typical and atypical mycobacteria have been involved in diseases of both human and lower animals. In humans these diseases are primarily caused by typical mycobacteria range from superficial infections of the skin to infection of the lungs caused by M. tuberculosis, and leprosy caused by M. leprae. In animals the disease is primarily found in cattle (M. bovis) chicken and birds (M. avium) and in rodents (M. lepraemurium). However, the atypical organisms have also been known to cause both skin (M. marinum), lung (M. kansasii) and at times lymph nodes (M. scrofulaceum) infections in humans and occasionally in certain animal species.

Mycobacterial Groups

Mycobacteria are divided into two main groups, typical and atypical. Typical mycobacteria are those that cause

clinical infections in man and animals. They become disseminated and may lead to the death of the host. In some instances the disease is self-healing. Typical infections can be transmitted by contact with the infected host. Atypical mycobacteria cause only restricted infections that are always self-healing and are not transmitted by contact with infected hosts. The members of the atypical group were divided in 1954 by Timpe and Runyon into four groups according to their response to light and growth temperature.

The objectives of this research are to separate the various protein antigens of unknown and known mycobacterial strains, both typical and atypical by isoelectricfocusing and chromatofocusing, and to evaluate their activity by ELISA in order to recognize both cross-reactive or shared as well as species specific characteristics. In addition, efforts have been made to place some of the unknown strains in the known spectrum of mycobacterial taxonomy.

CHAPTER II

REVIEW OF LITERATURE

Antigens, derived from mycobacteria, have probably been more widely used by clinicians and immunologists than any other microbial antigen preparations. Moreover, there has probably been less understanding accompanying their use than has been true for any other widely used antigen preparation. Extracts of mycobacteria - and tuberculins are nothing more than the crudest of such extracts - contain many antigens of varying chemical composition. Some of these antigens are probably species non-specific and almost certainly contribute to the antigenic cross-reactivity. There is a great need for isolated, purified, and standardized mycobacterial antigens, and a considerable number of studies have been carried out with mycobacterial culture filtrates or cell extracts, in hopes of isolating individual, purified mycobacterial antigens. The last review devoted to the subject of mycobacterial antigens was published more than two decades ago by Boyden and Sorkin (1956). It is probable that the number of individual mycobacterial antigens that can be identified and named is limited only by the effort which an investigator wishes to expend in the development of

appropriate technology. Many investigators have used precipitin techniques in gels for the analysis of mycobacterial antigens. Techniques used have included single- and two-dimensional immunoelectrophoresis. Polyacrylamide gel electrophoresis is a useful analytical tool for discriminating mycobacterial antigens. Provided that the physical conditions of acrylamide gel concentration, pH and buffer, and current flow are standardized, it provides a technique of powerful resolution that is readily adaptable to the identification and nomenclature of individual antigens. Moreover, the resolving power of gel electrophoretic techniques has been greatly increased by the two-dimensional procedures introduced by, among others, Augier and Augier-Gibory (1969) and Wright and Roberts (1974). During the past two decades, physicochemical fractionation methods that carry relatively little risk of denaturation have become available and have been used in attempts to isolate mycobacterial antigens. The methods used have included ion-exchange chromatography, molecular-exclusion, isoelectric focusing, and zonal electrophoresis with or without the molecular sieving effect of an acrylamide gel supporting system. Salt or solvent solubility has often been used in combination with these techniques (Table 1).

Table 1. Selected Major Physicochemical Purifications of Mycobacterial Antigens. *

Methods	Products
Ethanol-acetic acid precipitation	Proteins A,B,C and D and polysaccharides I and II; none antigenically pure
DEAE-cellulose ion-exchange chromatography	Protein-and polysaccharide-rich antigenic fractions; none antigenically pure
Molecular-exclusion chromatography	Protein - and polysaccharide-rich antigenically pure
Paper curtain electrophoresis	Protein-and polysaccharides rich antigenic fractions; none antigenically pure
Acrylamide gel electrophoresis	Small quantities of isolated, purified antigenic proteins

* Adopted from Daniel and Janicki, 1978.

Table 1. Selected Major Physicochemical Purifications of Mycobacterial Antigens (Continued)

Methods	Products
Serial ammonium sulfate precipitation, gel filtration ion-exchange chromatography, zonal electrophoresis	Highly purified antigenic proteins with low yields
Alkaline extraction, ethanol precipitation, ion-exchange chromatography	Highly purified antigenic arabinogalactan and arabinomannan; nonantigenic mannan and glucan
Concanavalin A affinity chromatography	Purified antigenic arabinoglactan and arabinomannan
Immunoabsorbent affinity chromatography	Two highly purified protein antigens only

Numerous other reports document the utility of ion-exchange chromatography in fractionating mycobacterial products, although no more than partial purification has been achieved by this single technique. Bennedsen (1970) subjected an unheated culture filtrate and a saline cell extract of M. tuberculosis to chromatography on DEAE-Sephadex after preliminary precipitation with 80% saturated ammonium sulfate. Glenchur et al. (1965, 1966 and 1973) used DEAE-cellulose chromatography in a series of studies of pressure cell extracts and culture filtrates of M. tuberculosis. They initially subjected their materials to Sephadex G-25 chromatography and showed that antigenic activity was located in the first eluted exclusion peak. This peak almost certainly contained all of the proteins and polysaccharides present in their starting materials.

Navalkar (1971) and Navalkar et al. (1975 a and b) prepared a cell extract from M. leprae isolated from lepromas and fractionated this material by DEAE-cellulose chromatography. Three fractions, designated A, B, and C, were obtained. Fractions A and B were found to contain multiple protein and polysaccharide antigens. Only a singly protein antigen could be identified in fraction C. When used in delayed skin test and passive cutaneous anaphylaxis studies in guinea pigs sensitized with M. leprae and other mycobacteria, species specificity

was found in fraction C but not in fraction A and B. More recently Navalkar et al. (1980) used fused rocket immunoelectrophoresis to evaluate the antigenic relationship between M. vaccae and M. leprae. Payne and Daniel (1980) employed immunoabsorbent affinity chromatography for identification and preliminary purification of protein antigen apparently specific to M. kansasii.

Other preparative approaches based on molecular-size differences, such as density gradient ultracentrifugation, have had only limited application. There have been numerous attempts to apply electrophoresis for fractionation of mycobacterial antigens. Rhodes et al. (1957) used zonal electrophoresis on glass beads to partially purify the polysaccharide hemosensitin of M. tuberculosis culture filtrate. Merkal (1961) used continuous-flow paper curtain zonal electrophoresis to fractionate further material precipitated from culture filtrate of M. paratuberculosis with ethanol and acetic acid. Janicki and his collaborators (1972) used the same technique to fractionate culture filtrates of M. tuberculosis and then subjected the fractions obtained to subsequent study and analysis. Several investigators have combined two or more physicochemical separation techniques serially. This approach has yielded

preparations in which only single antigenic constituents can be identified, although the yield of these materials has generally been very small. Abe et al. (1972) used zonal electrophoresis and Sephadex G-200 gel filtration to fractionate a soluble extract of lepromatous nodules. Their data suggested that a polysaccharide antigen of M. leprae is shared with other mycobacteria and that a major protein, which also can be extracted from lepromata, is antigenic, is derived from M. leprae bacilli, and is not shared with other mycobacteria. The combination of gel filtration with Sephadex G-200, DEAE-Sephadex ion-exchange chromatography, and isoelectric focusing was used to fractionate culture filtrate of M. tuberculosis by Moulton et al. (1971). They obtained nine fractions, four of which had precipitinogens when studied by immunodiffusion with rabbit antisera, two giving only single lines.

Electrophoresis on acrylamide gels is a fractionation method that combines zonal electrophoresis with molecular sieving provided by the supporting gel. The degree of cross-linking of the gel can be varied by changing the acrylamide concentration, resulting in differing sieving effects. By this method, extremely high resolution is possible. Affronti et al. (1965). First applied

acrylamide gel electrophoresis to the fractionation of mycobacterial antigens. Roszman et al (1968) used this approach to fractionate culture filtrates of several mycobacterial species. Upon completion of electrophoresis, gels were sliced using a stained gel as a guide and the slices were extracted with buffer. Sufficient quantities of materials were recovered in this way to allow study with antisera, and in some eluates only single antigens were identified by immunodiffusion. One very anodal antigen was thought to be specific for M. bovis and M. tuberculosis on the basis of immunodiffusion reactions. Modifications in the basic acrylamide gel electrophoretic technique have increased its resolving power. Augier and Augier-Gibory (1969) and Wright et al. (1972) have introduced two-dimensional procedures for analytic purposes. Affronti et al. (1972) introduced the use of discontinuous gel gradients in this technique. Finally, Minden and Farr (1969) developed a preparative scale method for using gradient acrylamide gel electrophoresis to fractionate mycobacterial antigens. Affinity chromatography is a purification method based on highly specific, dissociable interactions between two macromolecules. It is among the mose versatile and powerful purification methods available in the field of immunochemistry, but substances are isolated on the basis

of molecular parameters very different from those used in standard physicochemical procedures. That is, molecules are recovered together that have a given biological activity in common, even though they may have very different physical properties. Thus, when the isolation is made from lysed or physically disrupted cell walls, it is reasonable to expect great physical heterogeneity. In general, affinity chromatography methods can be expected to produce little or no denaturation or less of antigenicity in contrast to harsh chemical extraction procedures. Molecular subunits or peptide or carbohydrate moieties, for example, can be expected to remain attached to their parent molecules. These subunits may be completely extraneous to antigenicity of product, or they may carry significant antigenic determinants. The need for purified and well-standardized antigen is great. Not only are they necessary for accurate clinical diagnosis of tuberculous infection, but also mycobacterial antigens serve as standards in the assessment of cell-mediated (T lymphocyte) immunological functions for a variety of clinical and fundamental biological situation. Truly purified and standardized highly specific mycobacterial antigens should contribute broadly to our knowledge of major biomedical problems.

CHAPTER III

MATERIALS AND METHODS

Materials

Mycobacterial Strains

The cell extracts of 30 unknown strains of mycobacteria were obtained from the International Working Group on Mycobacterial Taxonomy (IWGMT) through the courtesy of Dr. Chaparas, Bureau of Biologics, National Institutes of Health (NIH). M. kansasii, M. marinum, M. scrofulaceum, M. avium, M. intracellulare, M. fortuitum, M. vaccae, and M. smegmatis cell extracts were also obtained from the same source. The known and unknown strains were derived from isolates from infected tissue and grown in vitro in a synthetic medium; the cell extracts (CE) were prepared by subjecting the cells to sonication for a predetermined interval of $4^{o}C$.

Antisera

The antisera used for the evaluation of the antigenic relationships between the unknown and known strains of mycobacteria were also obtained from Dr. Chaparas. These antisera were prepared against M. vaccae, M. kansasii, M. smegmatis M. avium, M. intracellulare, and M. simiae.

Methods of Procedure

Isoelectric Focusing

The development of isoelectric focusing (IEF) represents a major advance in the field of high resolution separation of proteins and other amphoteric macromolecules. IEF is an equilibrium method in which proteins are segregated according to their isoelectric points in pH gradient. The gradients are formed by electrolysis of amphoteric buffer substances known as carrier ampholytes. IEF, by virtue of being an equilibrium methods, has built-in resolution which allows separations of all components with different pI values. Figure 1 gives the methodology adopted in these studies. Commercially prepared polyacrylamide gel (PAG) plates, at a pH range of 3.5-9.0 were used in the preliminary screening and at a pH range of 4.0-6.5 for subsequent evaluation. For the pH gradient of 3.5-9.0, the buffers used were 1/2 M NaoH as the catholyte and 1M H_3PO_4 as the anolyte, whereas for the 4.0-6.5 gradient, 0.1M beta alanine was used as the catholyte and a mixture of 0.1M glutamic acid and 0.5M H_3PO_4 was used as the anolyte. After charging the respective cell extracts on a filter strip, IEF was run at constant current for a predetermined time. Marker proteins were used on the same gel for pI determination of each band. Thereafter the gels were fixed in a solution of TCA sulfosalicylic acid and water for 30 minutes. The plates

Figure 1. Isoelectric focusing protocol for mycobacterial antigens.

PAG plate (pH 3.5 - 9.5 or 4.0 - 6.5)

↓

Apply filter paper discs charged with cell extract

↓

Place wet electrode strips

Catholyte: 1M NaOH/3.5 to 9.5 and 0.1M Beta alanine/4.0 to 6.5

Anolyte : 1M H_3PO_4/3.5 to 9.5 and 0.1M Glutamic Acid in 0.5M H_3PO_4/4.0 to 6.5

↓

Run electrophoresis at constant current - 90 mins/3.5 to 9.5

(1200v; 12w; 30mA) 180 mins/4.0 to 6.5

↓

Remove PAG plate

↓

Immerse in fixative/1hr (3.0% Sulfosalicylic Acid + 10% TCA + H_2O)

↓

Transfer to destainer/10 mins (Ethanol + Acetic Acid + H_2O)

↓

Stain with warm Coomassie Brilliant Blue R-250/30 mins

↓

Destain to remove excess dye (several changes)

↓

Record

were then immersed in a destaining solution of glacial acetic acid, ethanol and water for additional 30 minutes and stained with Coomasie Blue R 250 overnight. Photographs were taken after the stained plates were destained to remove excess dye.

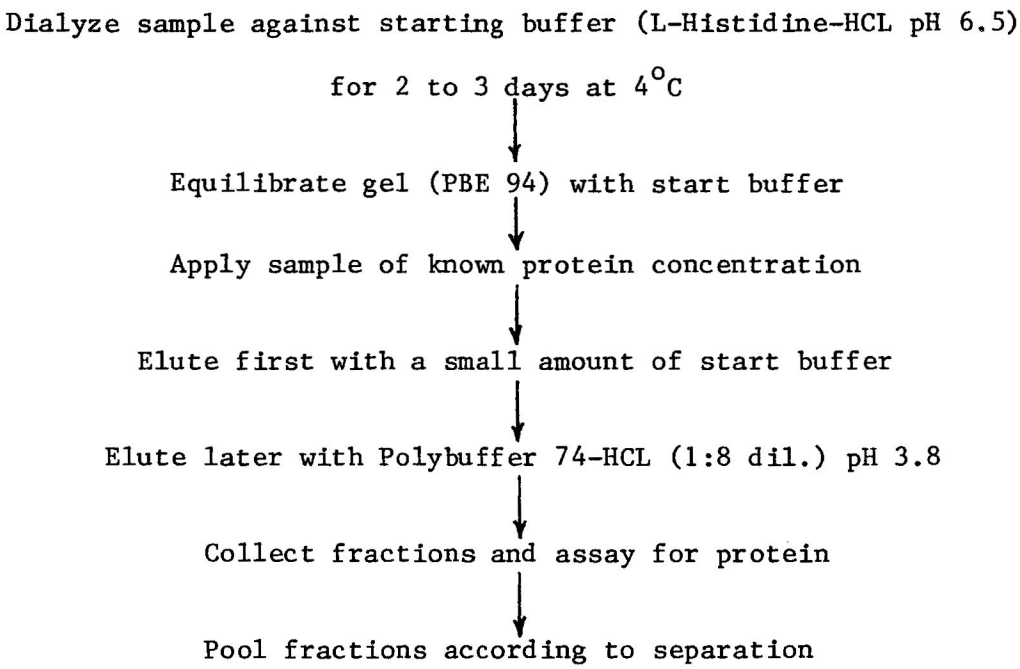
Chromatofocusing

Chromatofocusing technique, is a modified IEF, wherein the separation is effected in a column in which a pH gradient is produced on an ion exchanger. If a buffer at a given pH is run through an ion exchange column which was initially adjusted to second pH, a pH gradient is formed just as if the two buffers were gradually mixed in a mixing chamber of a gradient mixer. In addition to eluting proteins bound to the ion exchanger, focusing effects occur, which results in band sharpening, sample concentration and effective resolution. In these studies Polybuffer 96 with a pH range of 6-9 and polybuffer 74 with a pH range of 4-7 were used for chromatofocusing of mycobacterial cell extracts. Figure 2 gives the protocol for chromatofocusing of mycobacterial antigens.

Estimation of Proteins in Fractions

The protein concentration in each fraction, as well as the original cell extracts was determined by using Bradford technique (1976). Known protein solution containing 10 to 100 µg protein in a volume 0.1 ml was pipetted into 12 x 100 mm test tubes. Three milliliters of protein reagent was added to the test tubes, and

Figure 2: Protocol for chromatofocusing of mycobacterial antigens.


Dialyze sample against starting buffer (L-Histidine-HCL pH 6.5)
for 2 to 3 days at 4°C
Equilibrate gel (PBE 94) with start buffer
Apply sample of known protein concentration
Elute first with a small amount of start buffer
Elute later with Polybuffer 74-HCL (1:8 dil.) pH 3.8
Collect fractions and assay for protein
Pool fractions according to separation

the contents of each tube mixed by vortexing. The absorbance at 595 nm was measured after 10 minutes in 3 ml cuvettes against a reagent blank prepared from 0.1 ml of phosphate buffer with pH 7.2 and 3 ml of protein reagent. Bovine serum albumin, (BSA) cytochrome C, and ovalbumin were used for the preparation of the standard curve, and protein concentration in the various fractions was determined using the standard curve.

Elution of Antigen Material From PAG Plates

Each unknown and known mycobacterial strain was run at least three times in IEF using PAG plates with pH range of 3.5 to 9.0. Because of the similarity of the band patterns of many unknown strains, only four were chosen for detailed studies. These unknowns were 90053, 90066, 90068, and 90069. Seven known strains were used in addition to the four unknown ones for detailed studies. Since the band patterns of the known strains and the four selected unknown strains in the PAG plate with pH range of 3.5-9.0 were too closely arranged. PAG plates with a pH range of 4.0-6.5 were used to obtain better separation. Each selected unknown strain was run five times to obtain sufficient quantities of the band eluates. Using a stained gel of the same pH range as a guide, each band was sliced separately, and all the common bands of each unknown were collected in sterilized tubes containing sterile distilled water. The same procedures were followed with the seven known strains. After accumulating the

separate bands of each unknown and known strain, the contents of each tube were homogenized with a Teflon grinder in ice bath. The contents of each tube were then centrifuged at 1500 x g for 15 minutes. The supernatants were collected, and the pellets were discarded. Protein-containing supernatants were then concentrated by evaporation under constant air current. Following this the contents of each tube were reconstituted in phosphate buffered saline (PBS) with a pH of 7.2 for use in ELISA studies, since PBS is the vehicle used in ELISA analysis.

ELISA Studies

Commercially obtained polyvinyl chloride microtiter plates were used in these studies. Wells were coated 50 µl of each protein fraction of each unknown and known strains and incubated for 1 hour at 37°C. After that each well as filled with a 100 µl of 1% BSA, to block the remaining protein binding sites, and incubated overnight at 37°C. Each well was then washed 4 times with phosphate buffer saline (PBS) at a pH of 7.2. Immune serum from known strains 50 µl was added to each well except one, to which, 50 µl of normal rabbit serum was added as a negative control. The plates were then incubated at 37°C for 2 hours. Subsequently, wells were washed 4 times with PBS, and then 50 µl of peroxidase conjugated goat anti-rabbit IgG, was added and incubated for 1 hour at 37°C. Wells were washed 4 times with PBS and 100 µl of ABTS were added. The plates were

maintained at room temperature for 20 to 30 minutes. After 30 minutes, a green color developed in positive wells. 100 µl of stopping solution consisting of 5% SDS in 100 ml distilled water was added to stop the reaction prior to reading the plates. The data were recorded as 3+ for a very strongly positive reaction and graded to +/- and negative depending on the intensity of the reactions in subsequent wells.

CHAPTER IV

EXPERIMENTAL RESULTS

Isoelectric Focusing Studies

Initially, in all 37 mycobacterial strains were investigated, primarily on the pH 3.5 to 9.0 PAG plates. Of the 37 strains used, 7 were from known mycobacterial species and the remaining 30 were identified only by numbers allocated to them by the IWGMT. Table 2 gives the number of bands seen with these strains. Data comprise of several experiments conducted to confirm and reconfirm the distribution of the bands. Figures 3 through 7 give the actual photographs of the IEF runs of some of the strains used in these studies.

Main reason for using the 3.5-9.0 pH, a broad rang PAG plate, was to determine the distribution of the protein bands throughout the total pH spectrum. In no instance, were bands beyond the range of pH 5.5 noticed, thus indicating that all the proteins in each strain were of acidic nature.

Having recognized the fact that the distribution of the bands stayed within a limited range, use of PAG plates with the pH range of 4.0 to 6.5 was made. Primary reason for this was to recognize whether the closely associated bands seen in the 3.5-9.0 pH PAG plates, could be better separated so as to permit effective slicing of each band, if possible, an ultimately

Table 2. Results of Mycobacterial Cell Extracts Separated by IEF

Mycobacterial Strain	No. of Separated Bands	Mycobacterial Strain	No. of Separated Bands
90002	10	90053	8
90014	8	90054	6
90015	11	90056	9
90016	9	90057	7
90017	11	90060	10
90018	8	90061	9
90019	10	90062	7
90020	8	90066	11
90021	11	90067	11
90022	10	90068	8
90024	8	90069	10
90025	9	M.avium	11
90034	11	M.fortuitum	11
90037	10	M.marinum	9
90042	8	M.kansasii	10
90043	8	M.smegmatis	10
90045	11	M.scrofulaceum	8
90048	10	M.vaccae	8
90052	6		

Figure 3. Isoelectric focusing (IEF) of some IWGMT unknown strains on a broad pH range. The pH range of the gel was 3.5 - 9.0.

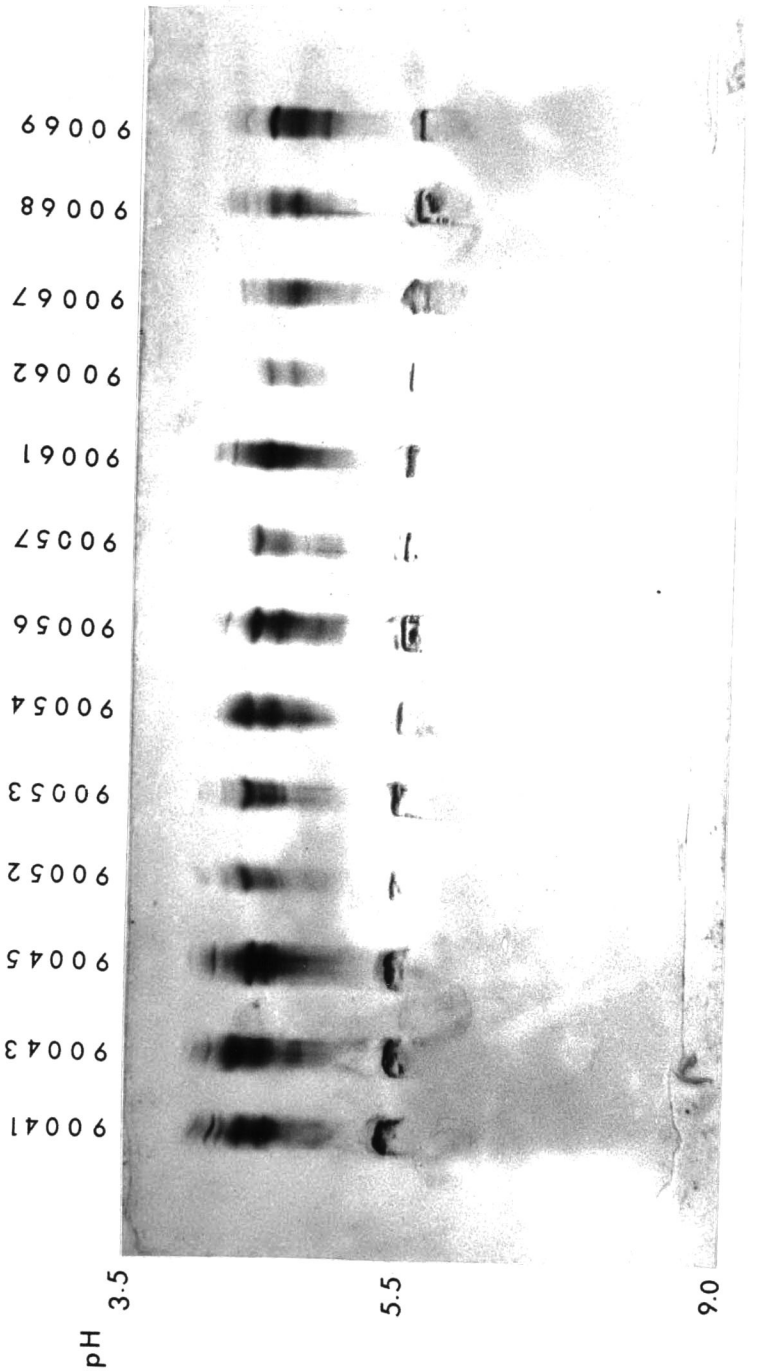

900641
900643
900645
900652
900653
900654
900656
900657
900661
900662
900667
900668
900669
pH
3.5
5.5
9.0

Figure 4. Isoelectric focusing of some IWGMT unknown strains on a limited pH range. The pH range of the gel was 4.0 - 6.5.

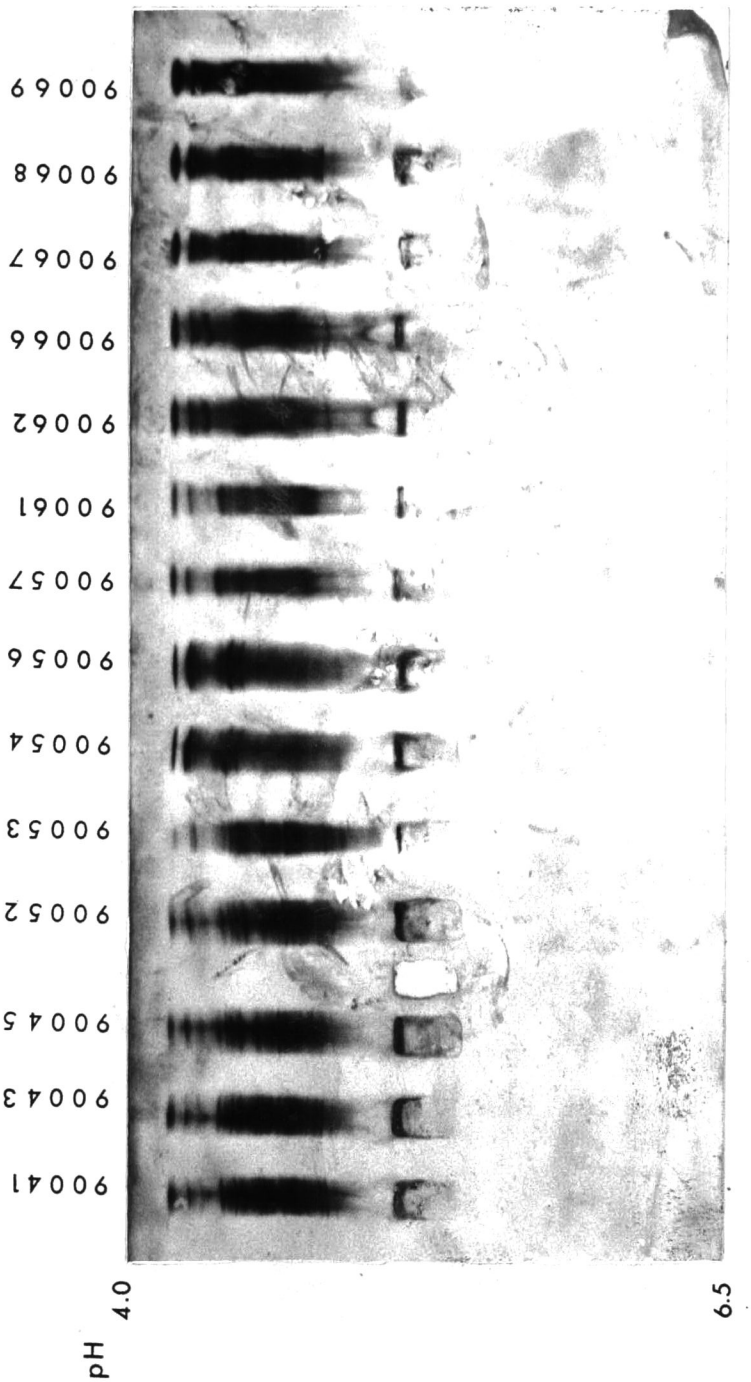

90069
90068
90067
90066
90062
90061
90057
90056
90054
90053
90052
90045
90043
90041
pH 4.0
6.5

Figure 5. Isoelectric focusing of the selected unknown IWGMT strains on a limited pH range. The pH range of the gel was 4.0 - 6.5.

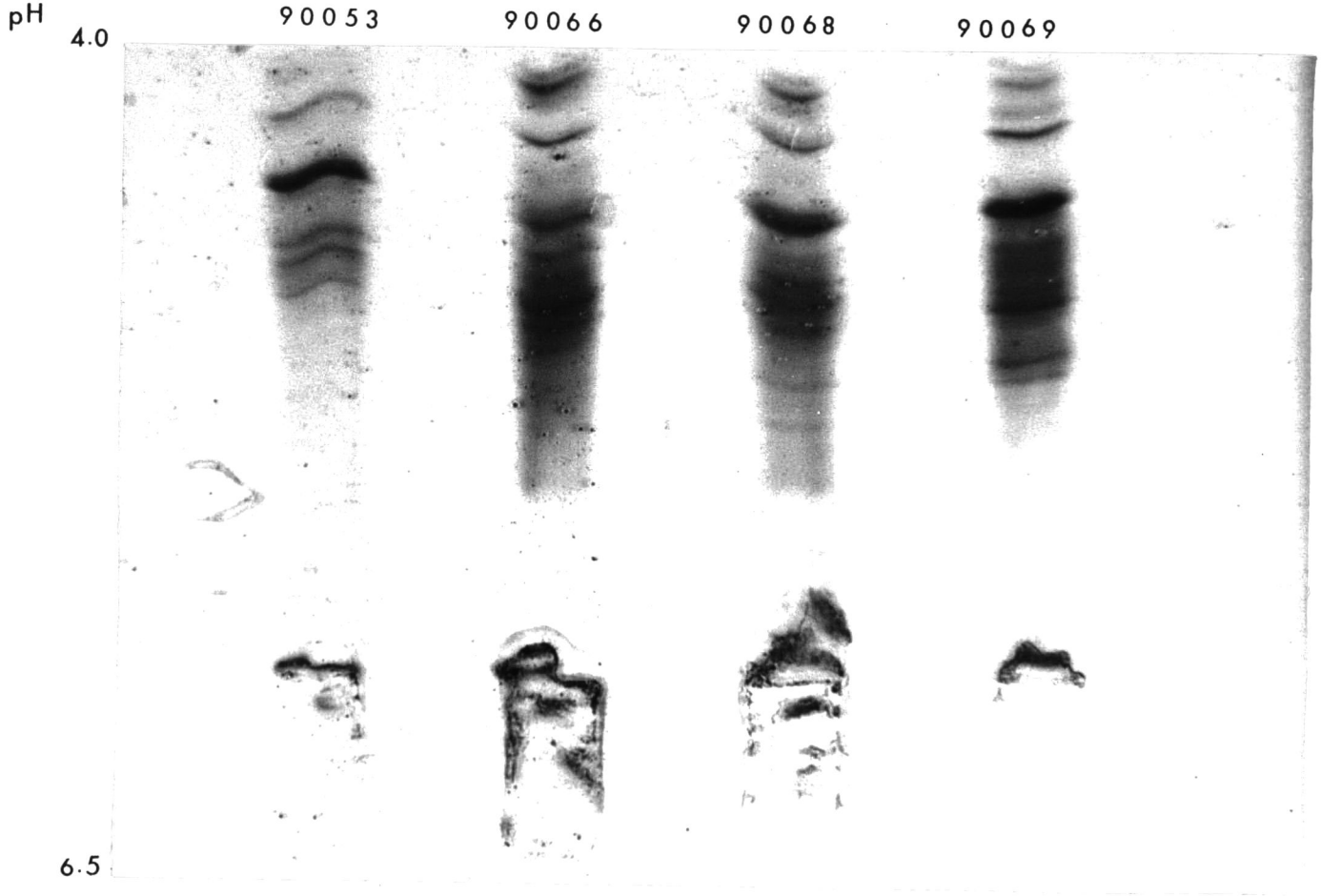

pH
4.0
90053
90066
90068
90069
6.5

Figure 6. Isoelectric focusing of some known mycobacterial strains on a broad pH range. The pH range of the gel was 3.5 - 9.0. A: M. kansasii; B: M. marinum; C: M. scrofulaceum; D: M. avium; E: M. intracellulare; F: M. fortuitum; G: M. vaccae H: M. smegmatis.

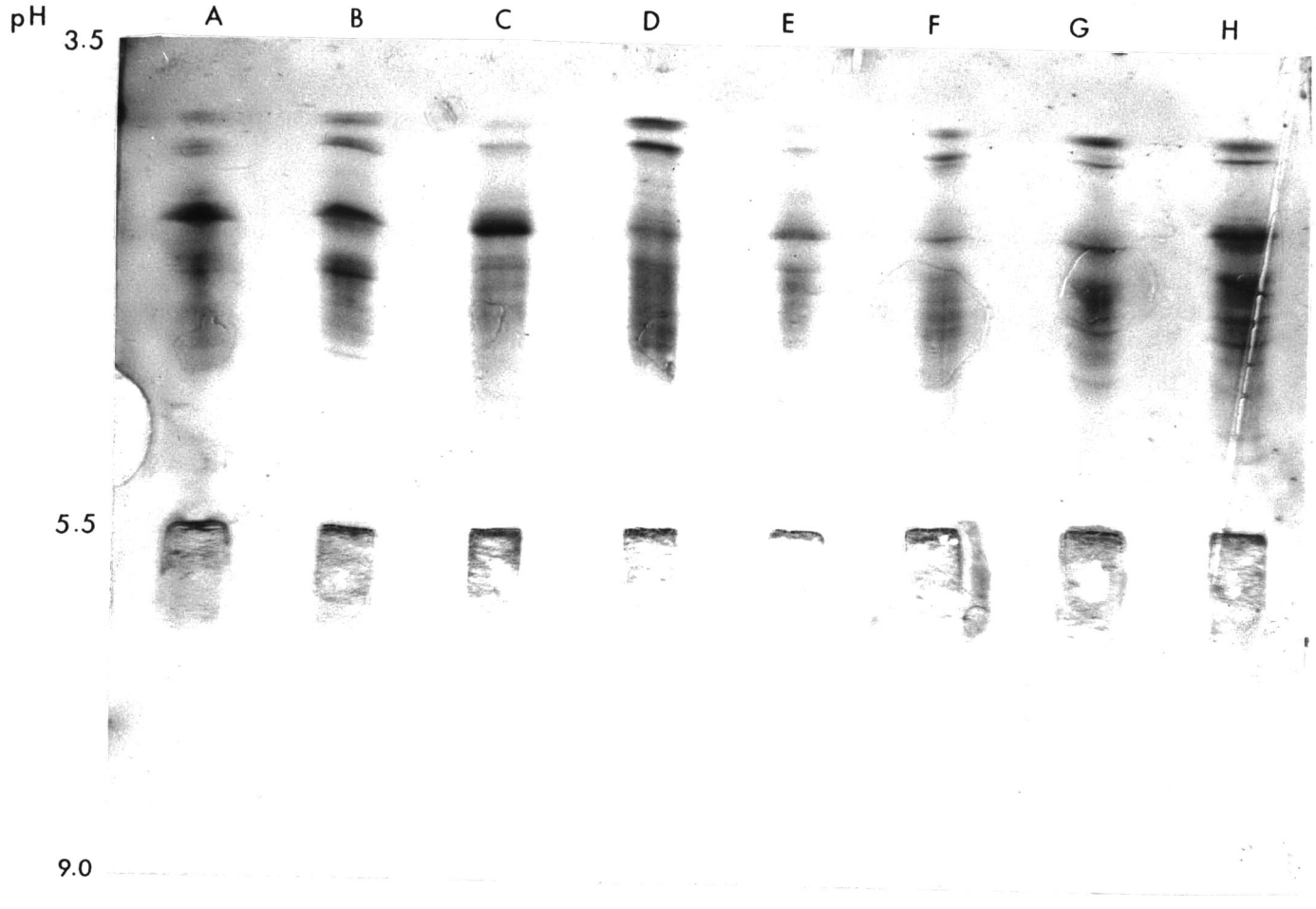
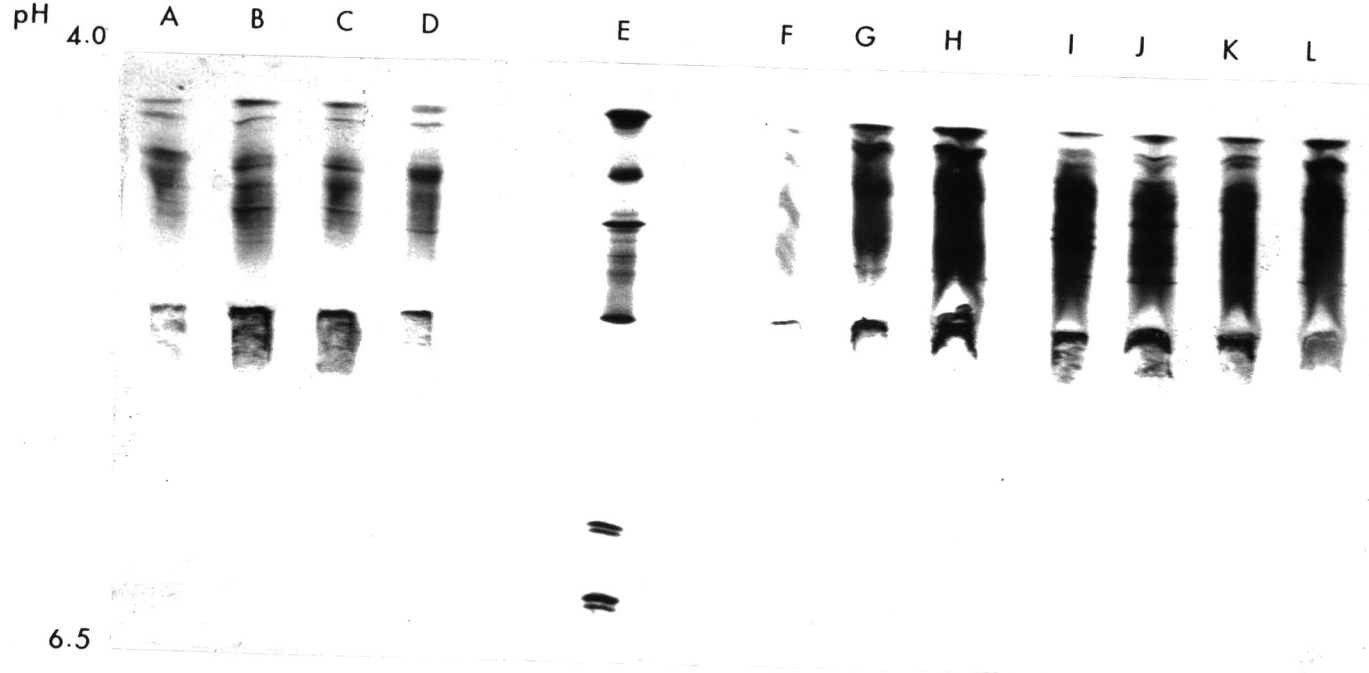

pH
3.5
5.5
9.0
A
B
C
D
E
F
G
H

Figure 7: Isoelectric focusing of the selected IWGMT known and unknown strains on a limited pH range. The pH range of the gel was 4.0 - 6.5. A: strain number 90053; B: strain number 90066; C: strain number 90068; D: strain number 90069; E: marker protein; F: *M. avium*; G: *M. fortuitum*; H: *M. smegmatis*; I: *M. scrofulaceum*; J: *M. kansasii*; K: *M. marinum*; L: *M. vaccae*.


pH
4.0
A
B
C
D
E
F
G
H
I
J
K
L
6.5

to extract the protein for immunological assays.

In both instances, i.e. with the 3.5 - 9.0 and 4.0 - 6.5 pH PAG plates, there was no change in the number of bands seen on either. Subsequently, it was decided to select only a few strains for further detailed studies since many of the strains exhibited identical band distribution as determined through Coomassie Brilliant Blue staining.

Table 3 gives the pI values of the different bands seen through IEF separation (pH 4.0 - 6.5) of the four IWGMT selected strains and the known strains. The isoelectric point (pI) for each band was determined using known markers at various pH ranges. The marker proteins used in these studies were Pepsinogen (pI 2.80), Amyloglucosidase (pI 3.50), Methyl red (pI 3.75), Glucose oxidase (pI 4.15), soybean trypsin inhibitor (pI 4.55) B-lactoglobulin A(pI 5.20), bovine carbonic anhydrase B (pI 5.85) and human carbonic anhydrase B (pI 6.55).

Chromatofocusing Studies

These studies were conducted only on the four selected strains. Figures 8 through 11, indicate the elution pattern of the proteins and their pH profile. As can be seen from these spectra, the distribution still restricts itself within the range of 3.8 to 6.5 pH, thus confirming the observations made on the IEF with the polyacrylamide gels.

Protein Estimation Table nos. 4 and 5 give the data on protein estimation of the various bands on IEF and the various fractions obtained through chromatofocusing, resepectively.

ELISA Evaluation

At the completion of the test each reaction was graded according to the intensity of color. With 3+ assigned to color development and +/- assigned to a very weak or faint color. As can be observed from the data obtained, the organism 90053 could be identified as M. simiae since all bands of this organism gave strong positive reactions against the M. simiae antiserum, the organism 90066 could be identified as M. avium since all bands of this organism gave strong positive reaction against the M. avium antiserum, the organism 90068 could be identified as M. vaccae since all bands of this organism gave strong positive reactions against the M. vaccae antiserum and the organism 90069 could not be identified with any of the mycobacterial systems studied, since it exhibited cross-reactivity throughout. It is likely that if many more systems had been used, this organism could have been identified. Studies on the individual known strains have confirmed the observation made above. Since when a given strain, such as M. smegmatis, was screened against its own antiserum, all bands gave positive ELISA reactions. This indicates that the unknown that gave all positive reactions with

a given known antiserum, must be the same organism as the known. It has also been possible to recognize some of the bands as specific to that particular organism. For example, bands 5, and 6 of M. avium could be specific antigens of M. avium and bands 6 and 8 of M. smegmatis could be specific antigens of M. smegmatis, since they do not show cross-reactivity with any of the other antisera used. A similar situation is seen with the unknowns, for example organism 90053 may possess the antigens in bands 1 and 8, which could be specific. In some instances, a very faint color bordering on the negative was observed. The possibility is that this weak color may not be due to a cross-reacting antigen does, however, exist and cannot, therefore, be overlooked.

Table 3. PI's of Various Mycobacteria

Mycobacterial Strain	Band Numbers (pI)										
	1	2	3	4	5	6	7	8	9	10	11
90053	2.80	3.00	3.00	3.50	3.50	3.50	3.75	4.15			
90066	2.80	3.00	3.50	3.75	4.15	4.55	4.55	5.15	5.15	5.55	5.55
90068	2.80	3.30	3.50	3.50	3.50	3.75	4.15	4.55			
90069	2.80	2.80	3.00	3.30	3.50	3.75	3.75	3.80	4.13	4.15	
M.avium	2.80	2.80	3.50	3.50	3.75	3.75	3.75	4.15	4.15		
M.fortuitum	2.80	3.20	3.20	3.50	3.50	3.75	3.75	4.15	4.15	4.55	4.55
M.smegmatis	2.80	3.30	3.30	3.50	3.50	4.15	4.15	4.55	4.55	4.15	
M.marinum	2.80	3.20	3.20	3.50	3.50	3.75	3.75	4.15			
M.scrofulaceum	2.80	3.50	3.50	3.75	3.75	4.55	4.55	5.20			
M.kansasii	2.80	3.20	3.75	3.50	3.75	4.15	4.15	4.55	4.55	5.20	
M.vaccae	2.80	3.50	3.20	3.55	3.75	4.55	4.55	5.20			

Figure 8. Chromatofocusing spectrum of IWGMT strain number 90053.

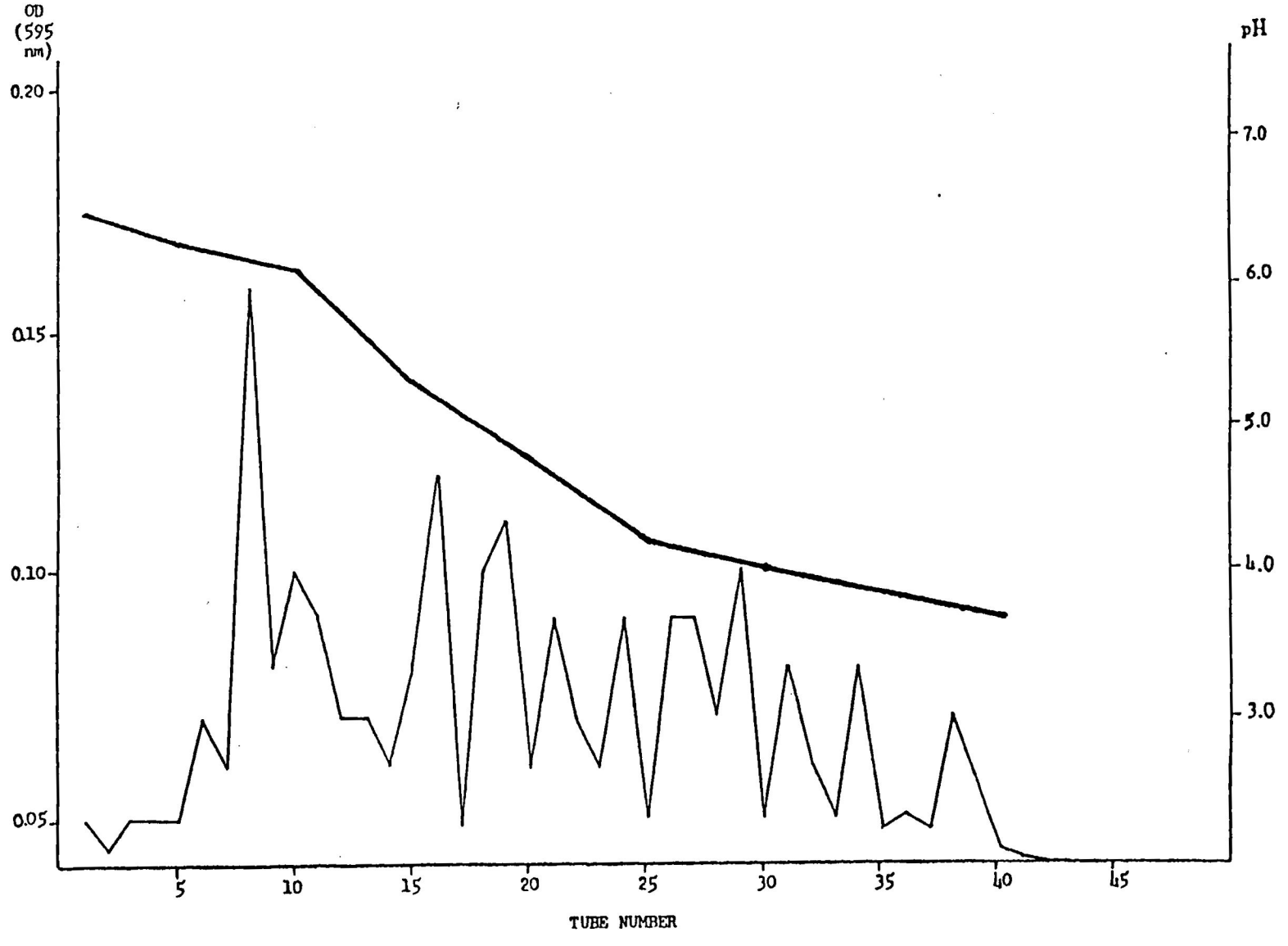

OD
(595
nm)
0.20
0.15
0.10
0.05
pH
7.0
6.0
5.0
4.0
3.0
5
10
15
20
25
30
35
40
45
TUBE NUMBER

Figure 9. Chromatofocusing spectrum of IWGMT strain number 90066.

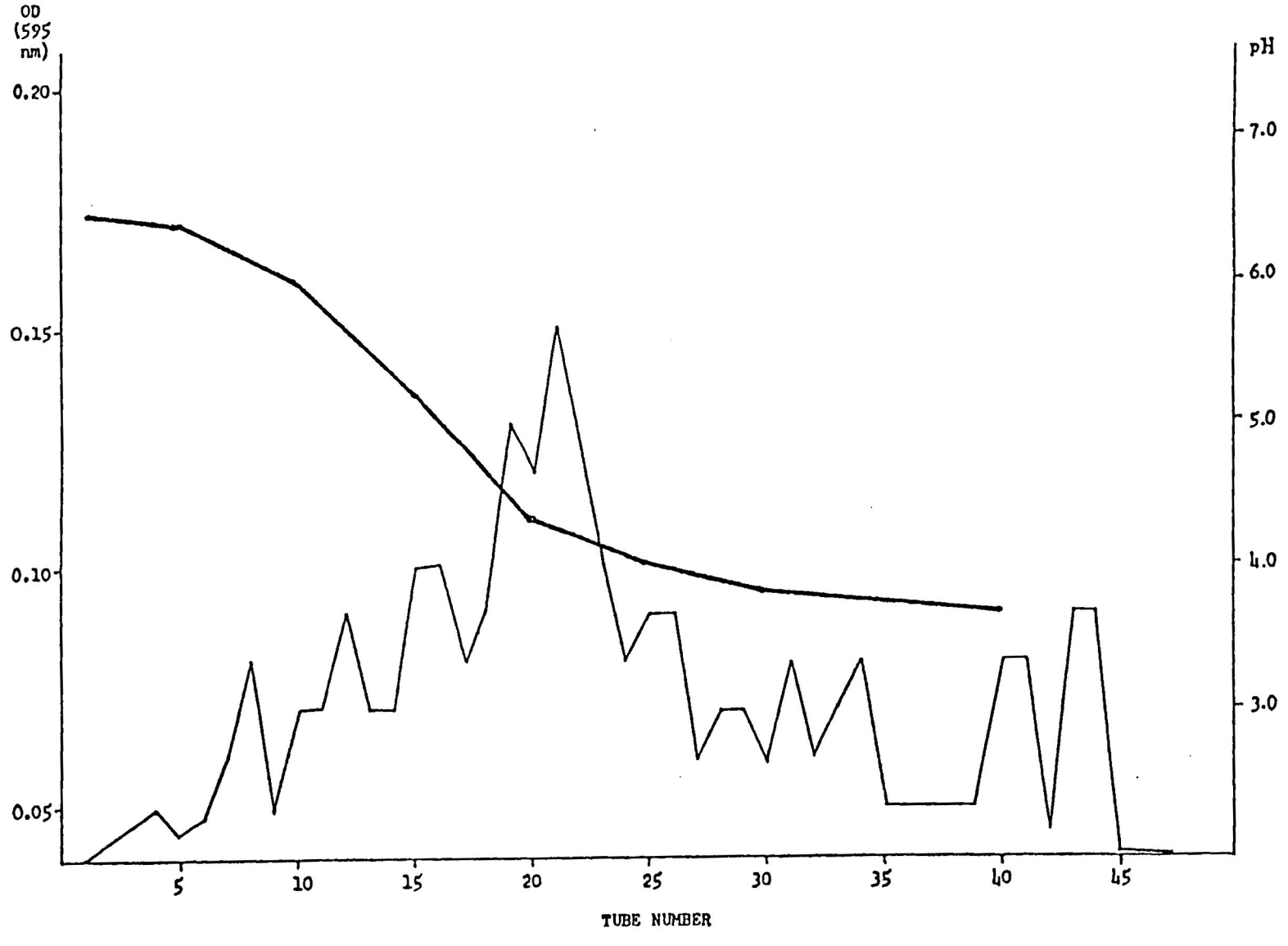

OD
(595
nm)
0.20
0.15
0.10
0.05
pH
7.0
6.0
5.0
4.0
3.0
5
10
15
20
25
30
35
40
45
TUBE NUMBER

Figure 10. Chromatofocusing spectrum of IWGMT strain number 90068.

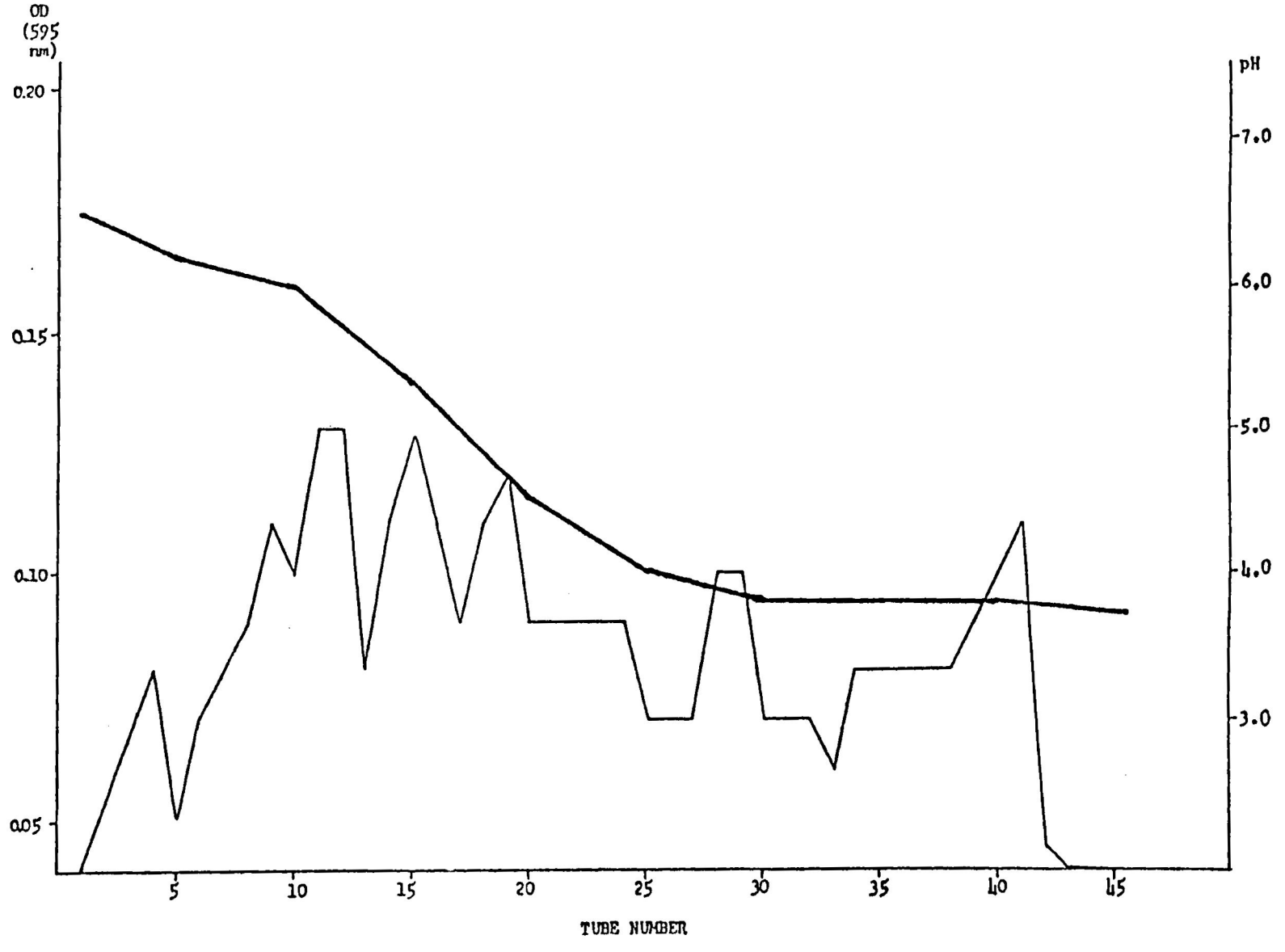

OD
(595
nm)
0.20
0.15
0.10
0.05
pH
7.0
6.0
5.0
4.0
3.0
5
10
15
20
25
30
35
40
45
TUBE NUMBER

Figure 11. Chromatofocusing spectrum of IWGMT strain number 90069.

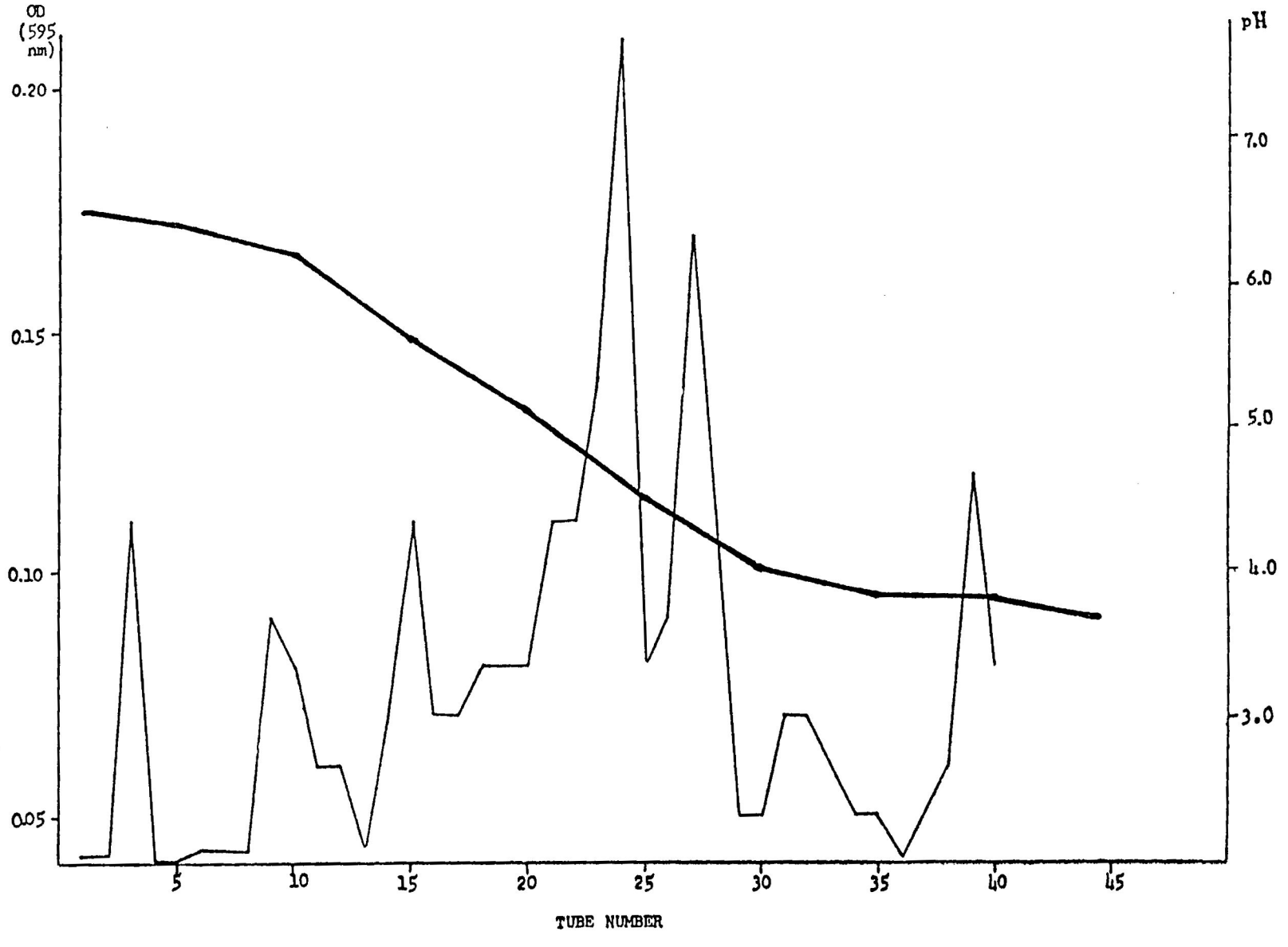

OD
(595
nm)
0.20
0.15
0.10
0.05
pH
7.0
6.0
5.0
4.0
3.0
5
10
15
20
25
30
35
40
45
TUBE NUMBER

Table 4. Protein Estimation of Various Bands Separated by IEF

Mycobacterial Strain	µg Protein/Band										
	1	2	3	4	5	6	7	8	9	10	11
90053(130 µg/ml)	62	42	51	48	70	50	45	51			
90066(200 µg/ml)	48	50	82	70	70	62	51	45	52	50	30
90068(190 µg/ml	32	66	75	65	66	66	70	65			
90069(150 µg/ml)	48	51	62	55	66	70	62	66	56	51	
M.avium(200 µg/ml)	20	48	50	50	48	51	20	24	32	50	20
M.fortuitum(200 µg/ml)	20	24	50	40	24	45	50	22	22	30	10
M.marinum(230 µg/ml)	35	42	50	52	40	51	45	30	25		
M.kansasii(260 µg/ml)	42	48	56	62	55	74	50	60	50	40	
M.smegmatis(215 µg/ml)	20	23	20	25	40	22	24	45	30	20	
M.scrofulaceum (230/ µg/ml)	35	42	40	23	52	48	30	20			
M.vaccae (260/ µg/ml)	10	30	10	20	15	20	10	10			

Table 5. Protein Estimation of the Various Fractions of the Four IWGMT Unknown Strains Obtained by Chromatofocusing

Mycobacterial Strain	µg Protein/Fraction				
	A	B	C	D	E
90053(130 µg/ml)	13	40	37	10	15
90066(200 µg/ml)	80	63	60	20	
90068(190 µg/ml)	13	52	30	20	15
90069(150 µg/ml)	17	40	52	60	27

Table 6. ELISA Reactions of the Various IEF Bands of the Four IWGMT Unknown Strains

Antisera against	90053 Band No.							
	1	2	3	4	5	6	7	8
M.vaccae	–	2+	2+	+/–	2+	2+	+/–	–
M.kansasii	–	–	–	+/–	–	–	+/–	+/–
M.smegmatis	–	–	–	–	–	–	–	–
M.avium	+/–	+/–	+/–	–	+/–	–	–	–
M.intracellulare	–	–	–	–	–	–	–	–
M.simiae	3+	3+	3+	3+	3+	3+	3+	3+

Antisera against	90068 Band No.							
	1	2	3	4	5	6	7	8
M.vaccae	3+	3+	3+	3+	3+	3+	3+	3+
M.kansasii	+/–	+/–	–	–	–	+/–	+/–	+/–
M.smegmatis	–	–	+/–	+/–	+/–	+/–	+/–	+/–
M.avium	–	–	–	–	–	–	+/–	–
M.intracellulare	–	–	–	–	–	–	–	+/–
M.simiae	–	–	–	–	–	–	–	–

Table 6. ELISA Reactions of the Various IEF Bands of the Four IWGMT Unknown Strains (Contd.)

Antisera against	90066 Band No.										
	1	2	3	4	5	6	7	8	9	10	11
M.vaccae	2+	+/-	+/-	-	-	-	-	+/-	-	+/-	-
M.kansasii	+/-	+/-	-	-	-	-	-	-	-	-	+/-
M.smegmatis	-	-	-	-	-	-	-	-	+/-	+/-	+/-
M.avium	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+
M.intracellulare	+/-	+/-	+/-	+/-	+/-	-	2+	2+	2+	+/-	+/-
M.simiae	-	-	-	-	-	-	-	-	-	+/-	-

Antisera against	90069 Band No.									
	1	2	3	4	5	6	7	8	9	10
M.vaccae	3+	+/-	+/-	+/-	-	-	-	-	-	-
M.kansasii	2+	+/-	+/-	2+	+/-	+/-	2+	3+	+/-	3+
M.smegmatis	-	-	-	-	-	-	-	2+	2+	-
M.avium	3+	+/-	+/-	-	-	-	-	-	+/-	-
M.intracellulare	2+	+/-	+/-	-	-	-	-	+/-	-	-
M.simiae	-	-	-	-	-	-	+/-	-	+/-	-

Table 7. ELISA Reactions of the Various IEF Bands Of The Four IWGMT Known Strains

Antisera against	*M.avium* Band No.										
	1	2	3	4	5	6	7	8	9	10	11
M.vaccae	2+	2+	+/-	+/-	-	-	-	-	-	+/-	+/-
M.kansasii	+/-	+/-	+/-	-	-	-	-	-	-	+/-	2+
M.smegmatis	-	-	-	-	-	-	-	-	-	+/-	+/-
M.avium	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+
M.intracellulare	+/-	+/-	+/-	+/-	+/-	-	3+	2+	2+	+/-	+/-
M.simiae	-	-	-	-	-	-	-	-	-	-	-

Antisera against	*M.vaccae* Band No.							
	1	2	3	4	5	6	7	8
M.vaccae	3+	3+	3+	3+	3+	3+	3+	3+
M.kansasii	-	+/-	-	-	-	-	-	-
M.smegmatis	-	-	+/-	+/-	-	+/-	-	+/-
M.avium	+/-	-	-	-	+/-	-	-	-
M.intracellulare	-	-	-	-	+/-	-	-	-
M.simiae	-	-	-	-	-	+/-	-	+/-

Table 7. ELISA Reactions of the Various IEF Bands of the Four IWGMT Known Strains (Continued)

Antisera against	*M.kansasii* Band No.									
	1	2	3	4	5	6	7	8	9	10
M.vaccae	2+	2+	+/-	+/-	+/-	-	-	-	2+	2+
M.kansasii	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+
M.smegmatis	-	-	-	+/-	+/-	+/-	2+	2+	2+	-
M.avium	2+	2+	+/-	-	-	-	-	-	+/-	+/-
M.intracellulare	2+	2+	+/-	-	-	-	2/+	2+	-	-
M.simiae	-	-	-	-	-	-	-	-	-	-
Antisera against	*M.smegmatis* Band No.									
	1	2	3	4	5	6	7	8	9	10
M.vaccae	+/-	+/-	+/-	+/-	+/-	-	-	+/-	+/-	+/-
M.kansasii	-	-	-	+/-	+/-	-	-	-	+/-	-
M.smegmatis	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+
M.avium	+/-	+/-	+/-	-	-	-	+/-	-	-	-
M.intracellulare	+/-	+/-	+/-	-	-	-	-	-	-	-
M.simiae	-	-	-	-	-	-	-	-	-	+/-

Table 8. ELISA Reactions of the Various Fraction of the Four IWGMT Unknown Strains Obtained by Chromatofocusing

Strain No.	Antisera against	Fraction Numbers				
		A	B	C	D	E
90053	M.vaccae	+/-	2+	2+	+/-	-
	M.kansasii	+/-	+/-	3+	2+	+/-
	M.smegmatis	-	+/-	+/-	-	+/-
	M.avium	+/-	+/-	-	-	-
	M.intracellulare	-	-	-	-	-
	M.simiae	3+	3+	3+	3+	3+

Strain No.	Antisera against	Fraction Numbers			
		A	B	C	D
90066	M.vaccae	2+	+/-	+/-	+/-
	M.kansasii	2+	+/-	-	-
	M.smegmatis	+/-	-	+/-	+/-
	M.avium	3+	2+	2+	2+
	M.intracellulare	+/-	+/-	2+	+/-
	M.simiae	-	-	-	+/-

Table 8. ELISA Reactions of the Various Fractions of the Four IWGMT Unknown Strains Obtained by Chromatofocusing (Continued)

Strain No.	Antisera against	Fraction Numbers				
		A	B	C	D	E
90068	M.vaccae	2+	2+	2+	2+	2+
	M.kansasii	+/-	+/-	+/-	-	+/-
	M.smegmatis	+/-	+/-	+/-	-/	-
	M.avium	-	-	+/-	+/-	+/-
	M.intracellulare	-	-	-	+/-	-
	M.simiae	-	-	-	-	-
Strain No.	Antisera against	Fraction Numbers				
		A	B	C	D	E
90069	M.vaccae	2+	+/-	+/-	-	-
	M.kansasii	2+	+/-	+/-	+/-	+/-
	M.smegmatis	-	-	+/-	-	+/-
	M.avium	2+	+/-	+/-	-	+/-
	M.intracellulare	+/-	+/-	-	-	-
	M.simiae	-	-	-	+/-	+/-

CHAPTER V

DISCUSSION

Mycobacteria are a complex mixture of antigens. These antigens are protein, polysaccharide and lipid in nature and are extremely difficult to obtain as individual moities. Efforts made to obtain these antigens as single entities have met with only partial success, despite various techniques employed. Thus it has not been possible to identify one particular technique that would not only permit effective separation but would also enable taxonomic placement of unidentified clinical isolates.

Of the several methods used, such as ion-exchange chromatography, Sephadex and DEAE Sephadex, acrylamide disc electrophoresis, isoelectric focusing and chromatofocusing now appear to be more promising than those used in earlier studies. Current studies using these two methods, although limited to a selected number of mycobacterial species, have indicated that the possibility of obtaining single antigens for immunological evaluation and for possible taxonomic assessment, may be a more realistic approach than otherwise expected.

The isoelectric focusing of the number of mycobacterial strains, both known and unidentified, has brought forth certain characteristics of the various protein antigens that form a part of the antigenic make up of these organisms. The fact

that all the protein bands for each of thirty seven strains evaluated, focus themselves within the pH range of 3.5 to 5.5, indicates that these antigens are acidic in nature. This characteristic has been found to be rather unique since other bacteria show a distribution of their protein antigen throughout the whole spectrum of 3.5 to 9.0 thus indicating the possession of antigens that are both acidic and basic in nature. In all previous studies, this situation was not recognized perhaps because of the techniques employed. These studies, therefore, have now added a new dimension to the understanding of the antigenic mosaic of mycobacteria.

Studies on chromatofocusing have further confirmed this observation, in addition to obtaining different fractions that can be used for immunological studies. In addition, the elution profile of each organism that has been studied both in this work and other extended work in our laboratories, is distinct and different. These spectra now allow, the possibility of identifying individual strains and placing them in their appropriate taxonomic position.

Isoelectric focusing, carried out on both the broad range (pH 3.5-9.0) and the limited range (pH 4.0-6.5) polyacrylamide gel plates, has shown that some of the strains studied exhibited quantitative differences in the number of bands although the

location was always within a restricted pH range. Of the number of strains evaluated many showed identical pattern of separation. Consequently, four representative strains were selected for extended studies. These were electrofocused on the limited range (pH 4.0-6.5) PAG plates several times and bands of individual protein antigens were sliced to obtain these in soluble form. The protein solutions of each band were pooled and concentrated. The concentrates were used to perform ELISA studies to determine antigen activity.

Immunological evaluation of the IEF band and the various fractions obtained through chromatofocusing for each strain, using the ELISA technique for the determination of the presence of antigen, has indicated the possibility of identifying the unknown strains studied. It now appears that in addition to detecting the common and shared antigens it is also possible to place the unknowns in either a mycobacterial cluster such as the well recognized MAI (Mycobacterium avium-intracellulare) group or identify them as known strains, as for example Mycobacterium simiae. At least one of the strains has identical positive reactions of all its bands and fraction with M. simiae.

One of the major problems that was faced was the effective separation of each of the bands in the IEF studies, since many

of them were closely placed. Slicing the gel, although carried out very carefully, could have contaminated the sliced band with some protein from the next band, thus resulting in crossreactivity. However, the results obtained from the chromatofocusing fractions indicate that this possibility was remote although not improbable. The results of the ELISA studies can therefore be considered as an indication of the use of either or both the IEF and chromatofocusing techniques as useful tools in the identification and characterization of the various mycobacterial antigens in addition to aiding in the taxonomic placement of a clinical isolate.

These studies have further significance, namely that each antigen-active fraction can now be assessed for its biological activities in respect of elicitation and/or induction of specific and non-specific delayed type hypersensitivity as well as its possible protective activity. Studies, currently being conducted in this laboratory on the separation and evaluation of the different antigens of Mycobacterium leprae have indicated that the fractions obtained through chromatofocusing have the capabilities of eliciting both the specific and crossreactive type of delayed hypersensitivity. The studies reported here have further afforded the opportunity to screen the various fractions for such evaluation.

CHAPTER VI

SUMMARY

These studies have indicated that all mycobacteria studied possess antigens that are acidic in nature as determined by the location of the protein bands on IEF with the pH range of 3.5 to 5.5, unlike other organisms. This appears to be a unique property of mycobacteria. In addition, the immunological analysis of the various antigen bands obtained by IEF and the various fractions obtained through chromatofocusing have now permitted the identification of specifc and shared antigens and have allowed identification of some of the unclassified strains.

These studies have also opened up the possibility of evaluating the biological potential of the different antigens from the point of view of elicitation of delayed type hypersensitivity induced in patients through infections with these strains, thus permitting identification of the causative organism, because of the specific nature of some of the separated antigens.

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